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Full Length Research Paper

Lercanidipine effect on polymorphonuclear leukocyte-related inflammation and insulin resistance in essential hypertension patients

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Inflammation, insulin resistance and oxidative stress (OS), are among the mechanisms that have been implicated in pathogenesis of essential hypertension (EH). Peripheral polymorphonuclear leukocytes (PMNLs) are primed in EH patients, releasing uncontrolled superoxide anion contributing to OS in these patients. PMNL priming correlates with insulin resistance and with PMNL intracellular calcium ($[Ca^{2+}]_i$). Recent studies have attributed to the anti-hypertensive drug lercanidipine, a third generation calcium-channel blocker, and additional anti-ischemic and anti-oxidative characteristics. To evaluate the possible non-traditional effect of two months of lercanidipine treatment on insulin resistance and on PMNL-related inflammation in EH patients. Non-smoking EH patients with untreated mild to moderate high blood pressure (BP) were included. Low-graded inflammation was reflected by WBC and PMNL counts and by PMNL apoptosis. Systemic inflammation was measured by plasma fibrinogen, C-reactive protein (CRP) and albumin levels. Fasting serum insulin levels served as a marker of insulin resistance. Two months of lercanidipine treatment showed significant decrease in BP, WBC and PMNL counts, and also in PMNL apoptosis, CRP and serum insulin levels and significant increase in serum albumin levels. Rates of superoxide release from PMNLs, WBC and PMNL counts and insulin levels positively correlated with mean arterial blood pressure values. We imply that use of lercanidipine can be favored in EH patients due to its combined anti-PMNL priming and anti-inflammatory effects, in addition to its anti-hypertensive characteristics.

Key words: Essential hypertension, lercanidipine, low-graded inflammation, primed polymorphonuclear leukocytes, oxidative stress.

INTRODUCTION

Essential hypertension (EH) is a substantial public health problem affecting 25% of the adult population in industrialized societies (Burt et al., 1995). This multi-factorial and multi-genetic disorder is a major risk factor for many common causes of mortality and morbidity, including stroke, myocardial infarction, congestive heart failure, and end stage renal disease (Mosterd et al., 1999). Insulin resistance is seen in more than half of patient with EH (Swislocki et al., 1989). Despite the important role of EH as a cause of disease, its pathogenesis remains largely unknown.

Abnormalities in endothelial function and morphology appear to play a central role in the pathogenesis of hypertension-related atherosclerosis (Zanchetti et al., 1993). Among the mechanisms causing endothelial dysfunction that have been recently implicated in EH, are OS that may impair endothelium-dependent vasodilatation,

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Abbreviations: **$[Ca^{2+}]_i$,** Intracellular calcium; **CCB,** calcium channel blocker; **DBP,** diastolic blood pressure; **EH,** essential hypertension; **MAP,** mean arterial pressure; **NC,** healthy subjects; **OS,** oxidative stress; **PMA,** phorbol 12-myristate 13-acetate; **PBS,** phosphate buffered saline; **PMNLs,** polymorphonuclear leukocytes; **SBP,** systolic blood pressure; **WBC,** white blood cells, **HD,** hemodialysis; **CKD,** chronic kidney disease.

Table 1. The changes in measurements of EH patients.

Ocular Fundus	HC	Untreated EH	1 month treatment	2 months treatment	*P*
	Negative	negative	negative	negative	
SBP (mmHg)	120±3.2[a]	162±4	146±3 [a]	143±3[a]	<0.01
DBP (mmHg)	69.1±2[a]	100±1	89±3 [a]	87±2[a]	<0.01
MAP (mmHg)	85.8±2[a]	120±2	108±2	107±2[a]	<0.01
Cholesterol (mg/dl)	205±1.7	230±9.3	225±13.2	226.3±15.6	ns
Triglycerides (mg/dl)	120±3.2	158±26	148.6±19.2	130±23.7	ns
HDL (mg/dl)	58±0.7	42.8±1.4	40.4±2	39.2±3.3	ns
LDL (mg/dl)	114±1.3	155±6.6	155.1±10.8	161±11.8	ns
Glucose (mg/dl)	94±1.9	89.1±4.5	98.4±3.4	100.8±5.0	ns
Creatinine (mg/dl)	0.93±0.02	0.96±0.03	0.98±0.04	0.95±0.04	ns
ALT (U/l)	20±0.5	38±6.2	30.7±6.3	38.4±5	ns
AST (U/l)	19.6±0.3	26.6±3.3	25.4±5.1	24±3.6	ns
ALP (U/l)	75±6.1	87.8±5.5	83.7±5.4	81.9±5.0	ns
LDH (U/l)	283±1.9	294±11.9	311±17	313±13	ns
Hb (g/dl)	14.3±0.1	14.7±0.25	14.6±0.3	14.7±0.2	ns
Insulin (U/ml)	8.4±0.9[a]	15.1±1.1	16.4±4.1	10.1±1.1[a]	<0.01

Values are means ± SEM, [a]versus untreated EH patients; ns = non significant.

tion, inflammation and insulin resistance (Alexander, 1995). Primed PMNLs are one of the main types of inflammatory cells; once activated, primed PMNLs release reactive oxygen species (ROS), contributing to OS, low-graded inflammation, endothelial damage and atherosclerosis in the long run (Smedly et al., 1986; Weiss, 1989).

Recently, we have previously reported that primed PMNL contribute to the OS and inflammation in correlation with insulin resistance and PMNL intracellular calcium ($[Ca^{2+}]_i$) in EH (Kristal et al., 1998; Sela et al., 2002a). In addition, we have recently implicated the PMNL priming as a key mediator of low-grade inflammation and OS associated with renal failure (Sela et al., 2005), thus constituting a common denominator in clinical states such as hypertension, renal failure, diabetes and cigarette smoking, known to be associated with endothelial dysfunction and accelerated atherosclerosis (Kristal et al., 1998; Sela et al., 2002b, 2005; Shurtz-Swirski et al., 2001).

The long-acting calcium channel blocker (CCB), widely used in the clinical setting have been shown to prevent atherosclerosis (Pitt et al., 2000; Tulenko et al., 2001; Hernandez et al., 2003), among which amlodipine has an antioxidative action *in vivo* (Napoli et al., 1999). In the current study, we examined the effects of the monotherapy lercanidipine, a vaso-selective dihydropyridine CCB that causes systemic vasodilatation by blocking the influx of calcium ions through L-type calcium channels in cell membranes as it is a highly lipophilic drug, as such it has a slower onset, longer duration of action and few adverse effects than a number of other CCB (Toyo-Oka and Nayler, 1996).

In a well-controlled clinical studies, once daily administration of lercanidipine 10 or 20 mg effectively reduced blood pressure compared with placebo in patients with mild to moderate hypertension without affecting heart rate (Bang et al., 2003). However, there are no precedent studies that demonstrate the various effects of this drug on systemic and PMNL-related inflammation and on insulin resistance during two months of treatment. Thus, the objective of the present study was to determine the effect of lercanidipine on these parameters in EH.

MATERIALS AND METHODS

Patients

Fifteen untreated EH patients (12 males/3 females), with mild to moderate hypertension (age range 20 to 65 years) and 15 age and gender-matched healthy controls (NC) were enrolled in this prospective study. Inclusion criteria of the EH group were: sitting diastolic blood pressure (DBP) > 90 mmHg (average of three outpatient visits), sitting systolic blood pressure (SBP) > 140 mmHg (average as above), body mass index < 30 kg/m^2, no evidence of target organ damage and systemic diseases supported by microalbumin/ceatinine ratio, fudus examination, echocardiogram test and kidney function tests. Subjects with evidence of acute or chronic infection, inflammation, receiving medication, vitamins or antioxidants, smoking and secondary causes of hypertension were excluded. The selection of all participants was based upon a clinical examination and laboratory confirmation. All the subjects had normal fasting (>14 h), serum cholesterol (<200 mg/dl), triglycerides (<150 mg/dl) and glucose levels, with normal kidney and liver function (Table 1). The study was approved by signing an informed consent for blood sampling approved by the institutional committee

in accordance with the Helsinki declaration.

Blood was drawn in the morning after an overnight fast from all EH patients and NC subjects for the determination of biochemical and hematological parameters and for PMNL isolation. Blood was drawn from EH patients before and following treatment with 10 mg/day lercanidipine (Vasodip™, Dexon, Israel) for 1 and 2 months. PMNL isolation was carried out from a 20 mL heparinized blood sample as previously described (Klebanoff and Clark, 1977; Sela et al., 2005). The separated PMNLs (>98% pure, approximately 10^7 cells per isolation) were resuspended in phosphate buffered saline (PBS) containing 0.1% glucose. Sera and plasma were frozen at -20°C for determining the clinical and biochemical characteristics of the participants and for systemic inflammation parameters.

PMNL priming

Rate of superoxide release

The measurements of the rate of superoxide release are based on superoxide dismutase (SOD) inhibitable reduction of 80 µM cytochrome C (Sigma, St. Louis, MO., USA) to its ferrous form (Babior et al., 1973). The rate of superoxide release was monitored from 10^6 separated PMNLs, after stimulation with 0.32×10^{-7} M phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO., USA), at 22°C for 50 min. This parameter was used as a measure for PMNL priming.

PMNL-derived inflammation

WBC and PMNL counts

Counts of WBC and PMNLs from blood drawn in Ethylenediamine-tetraacetic acid (EDTA) were performed by an automated cell counter (Coulter STKS, Miami, Fla., USA) and used as a measure of low-graded inflammation.

Analysis of apoptotic PMNLs

Apoptosis was analyzed in whole blood from EH patients and NC subjects of each group by flow cytometry according to Kuypers et al. (1996). Blood samples were assayed for apoptosis after lysis of red blood cells by Q prep (Beckman Coulter) and incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies using the Annexin V kit (Bender MedSystems, Vienna, Austria). PMNLs were defined by forward scatter/side scatter and by R-phycoerythrin (PE)-labeled monoclonal anti–CD16.

Systemic inflammation

Measurement of plasma fibrinogen

Fibrinogen was measured in a Cobas Mira plus instrument by Roche (Mannheim, Germany), in all plasma samples using the kit of K-Assay® (Kamiya Biomedical Company, Germany).

Measurement of C-reactive protein (CRP), transferrin and albumin

C-reactive protein (CRP), transferrin and albumin were routinely assayed in the routine biochemistry lab by Hitachi 917 Automatic analyzer (Roche Diagnostics, Mannheim, Germany) in separated sera obtained from all the above EH patients and NC subjects after an overnight fast.

Insulin as a marker of insulin resistance

Fasting serum insulin levels served as a measure for insulin resistance using the electrochemiluminescence immunoassay kit (Roche Diagnostics, Mannheim, Germany). Insulin resistance was also verified by homeostasis model assessment–insulin resistance (HOMA-IR) test.

Statistical analysis

Data values are means ± standard deviation (SD). The two groups were compared by student t-test, using Prism version 3.0 statistical software (GraphPad software, San Diego, California, USA). Correlations between different study parameters were performed using Pearson correlation coefficients. $P < 0.05$ was considered significant.

RESULTS

Study population

Table 1 summarizes the clinical and biochemical characteristics of the participants. All studied groups of patients showed similar serum cholesterol, serum creatinine, serum triglycerides, liver enzymes and serum glucose levels, without showing target organ damage. Most traditional risk factors were similar during lercanidipine treatment period. Blood pressure values, namely diastolic blood pressure (DBP), systolic blood pressure (SBP) and mean arterial pressure (MAP) decreased significantly following 1 and 2 months of lercanidipine treatment (Table 1).

PMNL priming

Rate of superoxide release

Significantly faster rates of superoxide release from PMA-stimulated PMNLs were found in EH patients before and following 2 months of lercanidipine treatment (Table 2), as compared to NC (18.2 ± 1.2 nmoles/10^6 cells/10 min), reflecting a higher priming state in these groups (EH). Two months of treatment reflected a slight though significant decrease in the rate of superoxide release from PMA-stimulated PMNLs (Table 2).

PMNL-derived Inflammation

WBC and PMNL counts

EH patients had significantly higher numbers of WBC and PMNLs (Table 2), as compared to NC subjects (6.7 ± 0.2 and $3.9 \pm 0.2 \times 10^9$, respectively), although all values fell within the upper quartile of the normal range. Two months of lercanidipine treatment reduced significantly WBC and PMNL numbers (Table 2).

Table 2. PMNL-related inflammation and priming and systemic inflammation parameters.

Parameter	HC	Untreated EH	1 month treatment	2 month treatment	*P*
WBC × 10^9	7.2±0.1	7.8±0.5	7.4±0.4	7.1±0.2[a]	<0.05
PMNL × 10^9	3.9±0.2[a]	4.8±0.4	4.4±0.4	4.2±0.2[a]	<0.05
PMNL apoptosis (%)	2.8±0.7[a]	15.4±1.8	11.5±2	7.2±1.0[a]	
Rate of superoxide release (nmoles/10^6 cells/10 min)	18.2±1.2	29±1.6	31.7±1.3	27.5±1.3[b]	<0.05
Fibrinogen (mg/dl)	289±12[a]	393±48	387±34	367±30	ns
Albumin (g/dl)	4.6±0.05[a]	4.5±0.06	4.6±0.07	4.64±0.05[a]	<0.05
Transferrin (g/dl)	273±5[a]	288±8	276±6	274±7	ns
CRP (mg/L)	1.5±0.08[a]	3.91±0.9	3.04±0.9	1.67±0.6[a]	

Values are means ± SEM. [a]versus untreated EH patients; [b]versus1 m lercanidipine treated EH patients.

Percentage of apoptotic PMNLs

The percentage of apoptotic PMNLs, assayed immediately after blood withdrawal in whole blood, was significantly higher in EH group of patients (Figure 1), as compared to NC (7.7 ± 0.4%). One month of lercanidipine treatment reduced significantly the percentage of apoptotic PMNLs, a reduction that further amplifies after 2 months of treatment, down to NC levels (Figure 1).

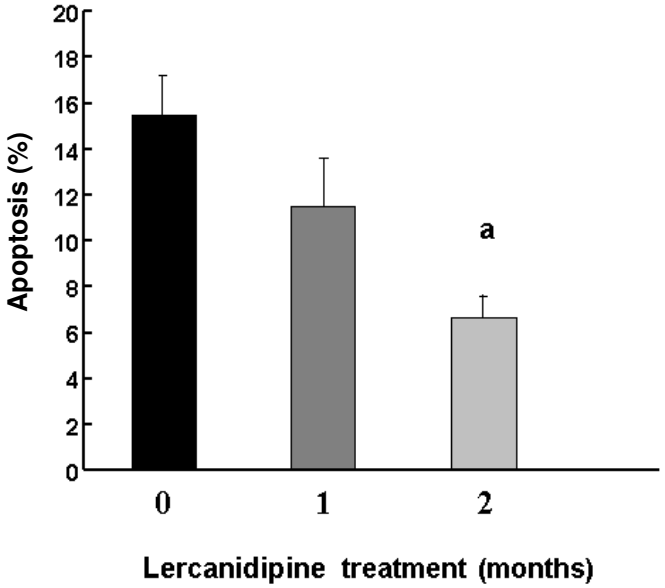

Figure 1. PMNL apoptosis in whole blood of EH patients, before and following 1 and 2 months of lercanidipine treatment. PMNL apoptosis was measured by flow cytometry using the Annexin V kit. Data are mean ± SEM. **a** = *P* = 0.001 versus PMNLs from untreated EH patients.

Systemic inflammation

Measurement of plasma fibrinogen

Plasma fibrinogen levels fell within the upper quartile of the normal range and were higher than NC levels (289 ± 12.3 mg/dl). A slight non-significant reduction in plasma fibrinogen levels was found after 2 months of lercanidipine treatment (Table 2).

Measurement of C-reactive protein (CRP), albumin and transferrin

A significantly decreased serum CRP levels could be shown after 2 months of lercanidipine treatment to NC levels (1.46 ± 0.08 mg/l) (Figure 2). Increased serum albumin levels were found in HC and treated EH patients compared with untreated EH patients, although all fell within normal range. No significant change was found in serum transferrin levels (Table 2).

Insulin as a marker of insulin resistance

Fasting serum insulin levels serve as a measure for insulin resistance (Sela et al., 2002). Figure 3 shows a significant decrease of serum insulin levels after 2 months of lercanidipine treatment, although this was still higher than NC levels (8.41 ± 0.95 µU/ml) after one month of treatment. It has to be emphasized that in these mild to moderate untreated EH patients, most serum insulin levels were within the normal range, although in the upper quartile.

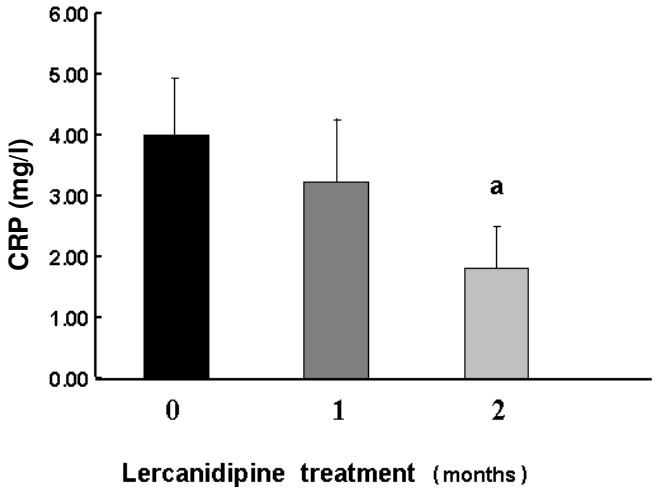

Figure 2. Serum CRP levels EH patients before and following 1 and 2 months of lercanidipine treatment. Serum CRP levels were routinely assayed in the routine biochemistry lab in separated sera obtained from all the above mentioned EH patients. Data are mean±SEM. a = *P* = 0.001 versus sera from untreated EH patients.

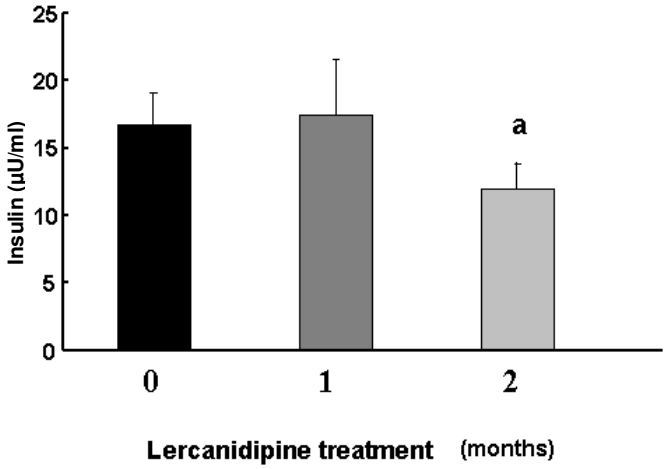

Figure 3. Serum insulin levels EH patients, before and following 1 and 2 months of lercanidipine treatment, using the electrochemiluminescence immunoassay kit. Data are mean ± SEM. **a** = *P* = 0.004 versus sera from untreated EH patients.

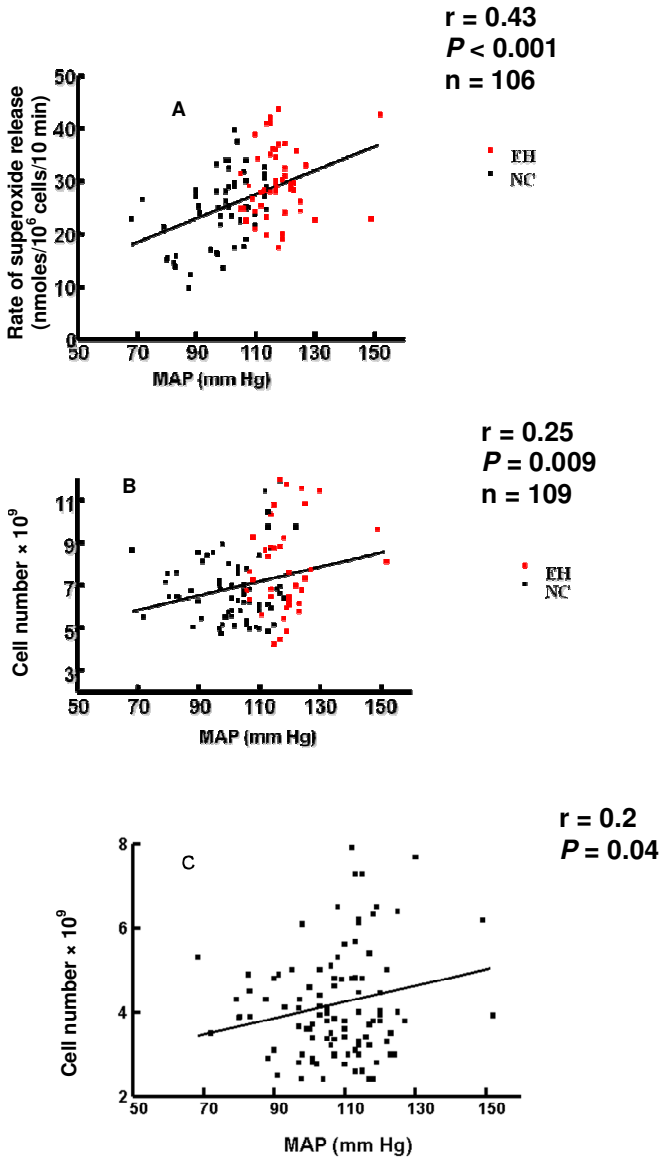

Figure 4. (A) Correlation between the rates of superoxide release from separated PMA-stimulated PMNLs and MAP; (B) Correlation between WBC counts and MAP; (C) Correlation between PMNL counts and MAP. Data refers to values from all EH patients (treated and untreated) and NC subjects (n = 106).

PMNL priming and inflammation in relation to MAP

PMNL priming expressed by the rate of superoxide release in NC and EH patients (treated and untreated with lercanidipine) was positively correlated with MAP: r = 0.45, *P* < 0.001 (n = 106, Figure 4A), the higher the blood pressure parameter, the higher the superoxide release. The WBC counts from NC and EH patients (treated and untreated with lercanidipine) were positively correlated with MAP: r = 0.25, *P* = 0.009 (n = 90, Figure 4B). The peripheral PMNL counts from NC and EH patients (treated and untreated with lercanidipine) were also positively correlated with MAP: r = 0.2, *P* = 0.04 (n = 109, Figure 4C).

Systemic inflammation parameters in relation to MAP

Fibrinogen and CRP, the accepted positive systemic inflammation markers, determined in NC and in EH patients (treated and untreated with lercanidipine), were correlated with MAP. Plasma fibrinogen levels positively correlated with MAP: r = 0.27, *P* = 0.007 (n = 101, Figure 5A). However, no correlation could be found between serum CRP levels and MAP: r = 0.05, *P* = 0.6 (n = 109,

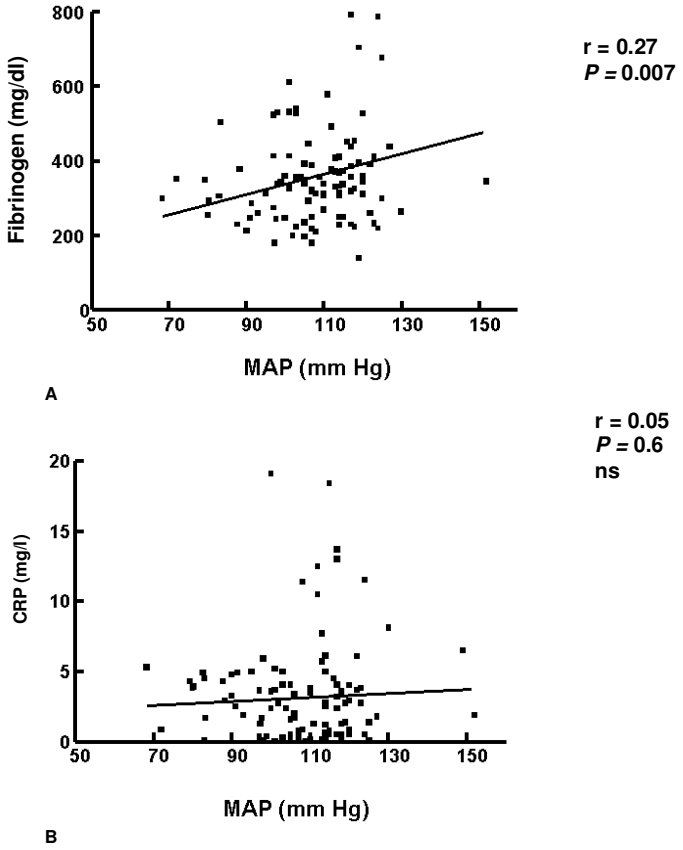

Figure 5. (A) Correlation between plasma fibrinogen levels and MAP; (B) Correlation between serum CRP levels and MAP. Data refers to values from all EH patients (treated and untreated) and NC subjects (n = 106).

Figure 5B).

Serum insulin levels in relation to MAP

Fasting serum insulin levels, serving as a measure for insulin resistance, were positively correlated with MAP: r = 0.36, *P* = 0.001 (n = 110, Figure 6).

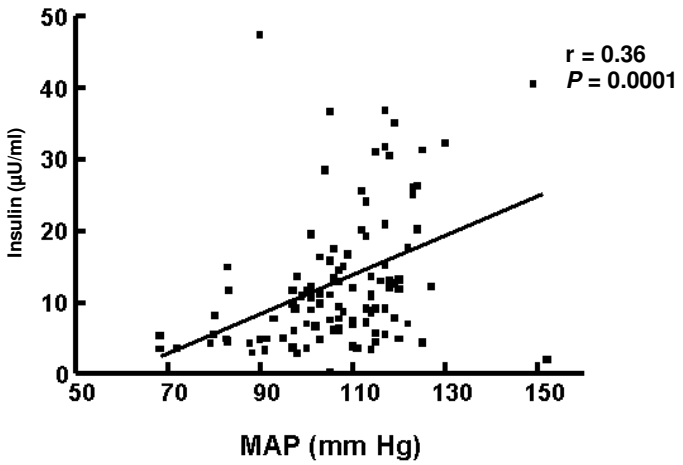

Figure 6. Correlation between serum insulin levels and MAP. Data refers to values from all EH patients (treated and untreated) and NC subjects (n = 106).

DISCUSSION

The present study evaluates the role of lercanidipine, a dihydropyridine CCB, in mild and moderate hypertensive patients and his non-traditional effects on PMNL priming, PMNL-related inflammation, systemic inflammation markers and on insulin resistance. Our previous studies showed that EH is accompanied by a primed state of PMNLs, inducing OS and inflammation (Kristal et al., 1998; Sela et al., 2005). We have defined PMNL priming as a common denominator in other clinical states such as hypertension, diabetes and cigarette smoking known to be associated with endothelial dysfunction, accelerated atherosclerosis and increased prevalence of cardiovascular morbidity and mortality (Kristal et al., 1998; Shurtz-Swirski et al., 2001; Tulenko et al., 2001; Sela et al., 2005). In addition, we have recently shown that PMNL priming constitutes a key mediator of low-grade inflammation and OS associated with renal failure (Sela et al., 2005).

A novel interesting observation was the significantly higher percentage of the apoptotic PMNL in EH patients as compared to normal control and the significant decrease in percentage of apoptotic PMNLs already one month after treatment with lercanidipine, a reduction further amplifies after two months of treatment to NC level. PMNL apoptosis has already been shown by us as part of PMNL-related low-grade inflammation parameters, along with WBC and PMNL counts (Sela et al., 2005), which constitute a mortality predictor in HD patients (Pifer et al., 2002; Reddan et al., 2003) and as a predictor for developing CKD (Erlinger et al., 2003).

In the present study WBC and PMNL counts were higher in EH patients as well, and declined significantly after treatment with lercanidipine, exhibiting a reduction in the PMNL-related low-grade inflammation.

In parallel, other systemic inflammation markers as CRP, fibrinogen, transferrin and albumin were also assessed. Serum albumin is a negative acute-phase protein whose low level is attributed to inflammation (Tsirpanlis et al., 2005); although in the normal range, we could show a significantly increase following Lercanidipine treatment. The reduction in fibrinogen was slight and non-significant, and transferrin levels did not change, possibly due to the relative small number of the patients. An interesting observation from the present study is the significant decrease in CRP level during this treatment to low levels as observed in NC, which are predictive of reduced cardiovascular risk. Numerous studies have demonstrated that elevated CRP levels and upper

quintile of normal levels are highly predictive of an increased incidence of cardiovascular events in healthy males and females (Kuller et al., 1996; Koenig et al., 1999; Ridker et al., 2001). The low-graded inflammation derived from PMNL priming does not correlate with CRP. These findings imply that different processes are involved in inflammation, which need to be further clarified.

In the present study, lercanidipine treatment significantly lowered fasting serum insulin levels. EH patients have higher plasma insulin levels in response to glucose load, whether obese or of normal body weight (DeFronzo and Ferrannini 1991). This hyperinsulinemia is a consequence of resistance to the effects of insulin on peripheral glucose utilization and to decreased hepatic uptake of insulin (Reaven and Laws, 1994). Elevated $[Ca^{2+}]_i$ has been described in various cells in insulin resistant states such as uremia, diabetes and EH (Ware et al., 1989; Draznin, 1993; Ohno et al., 1996). We have previously described in EH PMNLs a link between PMNL $[Ca^{2+}]_i$, plasma insulin and EH, adding PMNLs to previously described cells exhibiting elevated $[Ca^{2+}]_i$, contributing to OS and inflammation (Sela et al., 2002). Furthermore, the reported correlation of individual blood pressure with both PMNL $[Ca^{2+}]_i$ and plasma insulin levels, together with the fact that elevated PMNL $[Ca^{2+}]_i$ mediates PMNL priming, suggest that elevated PMNL $[Ca^{2+}]_i$ and insulin are involved in the pathogenesis of hypertension-induced vascular injury in EH. The cause of slight increases in glucose value of normal upper limit after 2 months treatment, not exactly known, this could be related to high activity of the enzyme hormone sensitive lipase (HSL) due to low concentration of insulin (Claus et al., 2005), but follow-up after several months did not develop diabetes or pre-diabetes in any of the participant patients. We showed in this study a correlation between MAP and PMNL-related priming and inflammation parameters. A significant link between blood pressure and ROS formation by PMNLs has been observed by Yasunary et al. (2005).

In addition, they reported an inhibition of ROS formation by PMNLs by treatment with benidipine, a long acting CCB, which can be attributed in part to the decreased blood pressure. However, they did not completely rule out the possibility that the drug itself served as an antioxidative agent (Yasunari et al., 2005). In our study, lercanidipine was chosen for treating hypertension, as a long acting CCB, because it has high efficacy on mild and moderate hypertension, low incidence of adverse effects and good tolerability by most patients. Several studies demonstrated that lercanidipine shows anti-ischemic and anti-oxidative effects (Bellosta and Bernini, 2000; Bang et al., 2003; Tomlinson and Benzie, 2003; Farah and Shurtz-Swirski, 2008; Martinez et al., 2008) due to its ability to inhibit the growth of smooth muscle cell and their migration to the blood vessel wall, indicating a possible anti-atherosclerotic effects of the drug (Bellosta and Bernini, 2000; Wu et al., 2009), thus may be useful in the treatment of insulin resistant hyper-tensive patients.

In summary, lercanidipine, in addition to its effect as an antiypertensive drug, carries anti-inflammatory features improving most inflammation markers, systemic and PMNL-related, and can improve insulin sensitivity. The amelioration in the inflammatory parameters can be attributed in part to the decrease in blood pressure. *In vitro* future experiments are needed to find a direct effect of lercanidipine on PMNL-contributed low-grade inflammation.

Limitation of the study

The small sample and not randomized and UN blinded, because we had to select the appropriate patients with no smoking and no other chronic illness without any hypertensive treatment that may cause a change in inflammatory markers. It was not so simple to find patients who meet the criteria. Other limitation was the control group were those patients themselves at baseline. The examined drug has not been tested *in vitro*, and only small sample with positive results not yet published were present.

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